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Abstract

Background: Filaggrin is an epidermal protein that is crucial for skin barrier function. Up to 10% of Europeans and 5% of Asians carry at least one null allele in the filaggrin gene (*FLG*). Reduced expression of filaggrin in null allele carriers is associated with facilitated transfer of allergens across the epidermis. We hypothesized that these individuals may have increased transdermal uptake of endocrine disruptors, including phthalates.

Objectives: We investigated urinary excretion of phthalate metabolites and testicular function in young men with and without *FLG* loss-of-function variants in a cross-sectional study of 861 young men from the general Danish population.

Methods: All men were genotyped for *FLG* R501X, 2282del4, and R2447X loss-of-function variants. Urinary concentrations of 14 phthalate metabolites were measured, as well as serum levels of reproductive hormones and semen quality.

Results: Sixty-five men (7.5%) carried at least one *FLG* null allele. *FLG* null carriers had significantly higher urinary concentrations of several phthalate metabolites, including a 33% higher concentration of MnBP (95% CI: 16, 51%). *FLG* null variants were not significantly associated with reproductive hormones or semen quality parameters.

Conclusion: This study provides evidence that *FLG* loss-of-function allele carriers may have higher internal exposure to phthalates, possibly due to increased trans-epidermal absorption. *FLG* loss-of-function variants may indicate susceptible populations in which special attention to trans-epidermal absorption of chemicals and medication may be warranted.

Introduction

In human stratum corneum of the skin, protein-enriched corneocytes are embedded in a lipid-rich matrix, impeding egress of water and penetration of pathogenic microorganisms, allergens, and noxious chemicals. To maintain skin hydration, abundant intracellular filaggrin proteins are hydrolyzed into amino acids and their deiminated products, collectively referred to as the "natural moisturizing factors" (Gruber et al. 2011). Filaggrin deficiency due to the presence of one or more loss-of-function variants in the filaggrin gene (*FLG*) is observed in approximately 10% of lightly-pigmented Europeans and in a slightly lower proportion of Asians (Irvine et al. 2011). These variants cause ichthyosis vulgaris (Smith et al. 2006), which is characterized by xerosis, scaling, and keratosis pilaris as well as palmar and plantar hyperlinearity. Permeation of allergens seems to be increased in filaggrin depleted skin (Fallon et al. 2009; Gruber et al. 2011; Scharschmidt et al. 2009), and an increased risk of atopic dermatitis, asthma, rhinitis, food allergies and nickel sensitization is observed (Brown et al. 2011; Palmer et al. 2006; Thyssen et al. 2010). Clinical trials are currently being conducted to study whether primary prevention of atopic disorders is possible using topical therapy with moisturizers (Irvine et al. 2011).

Diesters of 1,2-benzenedicarboxylic acid, commonly referred to as phthalates, are man-made chemicals used in a wide range of consumer products including moisturizers and other cosmetics. While phthalates are rapidly metabolized and excreted in the urine following absorption, humans are continuously exposed by skin contact with e.g. cosmetics, fragrances, solvents and plastics (Wittassek et al. 2011), as well as through the diet and from inhalation. An inverse association between phthalate exposure and markers of testicular function has been demonstrated in some human studies (Meeker et al. 2009; Mendiola et al. 2011). Animal studies have established that certain phthalates act as endocrine disrupters, resulting in developmental

abnormalities of the male reproductive tract as well as inhibition of testicular testosterone production in pre- or perinatally exposed animals (Boberg et al. 2011; Gray, Jr. et al. 2000). Although the pathogenic effects of phthalate exposure on testicular function in humans is not yet clear, certain phthalates inhibit testosterone synthesis in cultured adult human testes, at concentrations estimated to be within the range observed in epidemiological studies (Desdoits-Lethimonier et al. 2012).

In this study, we addressed the question whether male *FLG* null carriers would have increased internal exposure to phthalate metabolites, and if so, whether this would affect testicular function.

Methods

Study population

All Danish men are called to a compulsory examination to determine fitness for military service the year of their 18th birthday. Men residing in the Copenhagen area were approached for participation in a study on semen quality on the day they went for this examination. A total of 881 men volunteered from 2007 to 2009 for a study on urinary phthalate excretion and testicular function (Joensen et al. 2012). Participation rate was approximately 30%, which is higher than other population-based semen quality studies (Jørgensen et al. 2002). Basic study details have previously been described (Jørgensen et al. 2012). The men underwent a physical examination with focus on reproductive development, completed a questionnaire, and gave samples of spot urine, semen and blood, in most cases all within one hour. Ejaculation abstinence period and time of blood sampling were registered. All urine, semen and blood samples were collected between 8.40 am and 12.30 pm (median 10.00 am). In 2011, *FLG* genotyping was carried out

according to a protocol for the present study hypothesis. DNA samples from 13 of the 881 men were missing or of poor quality, and for another seven men, not enough urine was left for analysis of osmolality, which was also carried out for this study. Finally, 861 men had complete data on *FLG* variant status and other main outcomes. All laboratory analyses were carried out on coded samples.

The research protocol was approved by the Danish National Committee on Biomedical Research Ethics (no. H-KF-289428). Participants gave written informed consent before participation.

Questionnaire

Questions included information on lifestyle factors (e.g. smoking and alcohol consumption) and medical history. The responses were reviewed with the participants to clarify missing or equivocal information. Participants provided information on use of prescription and non-prescription medication within the past three months, but were not asked to further specify the time of use. Thus, men reporting medicine intake within the last three months may not have taken any within the last 24 hours or more. Ethnicity was deduced from self-reported country of birth of the participant and his parents, and 83 men were categorized as other than European, i.e. they themselves or one or both of their parents were born outside the European countries (20 from African countries, 16 Asian countries, 4 from Greenland, 11 from Latin American countries, and 32 from Middle Eastern countries). Participants were asked to report any previous diseases, with focus on reproductive disorders. The questionnaire included a specific question about previous diagnosis of asthma and hay fever, but no specific questions about eczema, food allergies or use of moisturizers or other personal care products.

FLG genotyping

DNA was purified from blood samples and stored at -80°C until *FLG* genotyping, which was performed slightly modified according to a previously published method (Meldgaard et al. 2012). Briefly, three regions covering the mutations R501X, 2282del4, and R2447X of the *FLG* were asymmetrically amplified from genomic DNA by PCR using DNA tagged allele-specific primers. The obtained single-stranded PCR products were hybridized with MagPlex-C micro beads (Luminex, Austin, Texas) carrying the DNA tags as probes, and subsequently analyzed on a Bio-Plex 200 (Biorad, Hercules, California). Deviation from Hardy-Weinberg equilibrium was tested for all three *FLG* null variants using the free online calculator at the Online Encyclopedia for Genetic Epidemiology studies (http://www.oege.org/software/hwe-mr-calc.shtml). Participants were classified as "filaggrin null carriers" if they carried at least one of the null *FLG* alleles that were evaluated (R501X, 2282del4, or R2447X).

Osmolality analyses

Urinary dilution was assessed by measurement of urinary osmolality. This was done by freezing point depression method using an automatic cryoscopic osmometer (Osmomat® 030, Gonotec, Berlin, Germany). For every twelve samples measured, a standard urine pool was measured. Mean urinary osmolality for this standard pool (N = 73) was 0.83 Osm/kg with a relative standard deviation (RSD) of 1.63%. Concentrations of phthalate metabolites were adjusted for osmolality by dividing the measured metabolite concentration with the urinary osmolality (Osm/kg) of that sample and multiplying the result by the median osmolality of the population (0.86 Osm/kg). As urine density is close to 1 kg/L, 1 Osm/kg was assumed to equal 1 Osm/L. Only samples with phthalate metabolite concentrations > LOD were adjusted in this way. Samples < LOD were left unadjusted for osmolality.

Urinary phthalate metabolite analyses

Urine samples were collected in polyethylene cups. 15 mL was decanted to a 20 mL glass scintillation vial with the top packed with aluminum foil, and stored at -20°C until analysis. Samples were analyzed for content of 14 phthalate metabolites (Table 1) by liquid chromatography tandem mass spectrometry (LC-MS/MS) with preceding enzymatic deconjugation followed by solid phase extraction. Methods for sample preparation, standard solutions, quality controls, instrumental analysis, and general method validation have previously been described (Frederiksen et al. 2010). Urine samples were analyzed in 25 batches over 11 weeks. Each batch included standards for calibration curves and approximately 35 unknown samples plus 2 blanks, 2 urine pool controls, and 2 urine pool controls spiked with phthalate standards in low levels. The interday assay variation, expressed as the relative SD, was < 20% for all analytes except MCPP (22%) and MiNP (26%). Recovery of spiked control samples was > 90% for all analytes except MnBP (88%) and MCPP (85%). Limits of detection (LODs) were established as previously described (Frederiksen et al. 2010). Results below LOD were assigned a value of LOD/√2.

Concentrations of phthalate diesters were calculated by multiplying the measured molar urinary concentration with the molar weights of the respective diesters. The sum of the two DBP isomers ($\Sigma DBP_{(i+n)}$) and the sums of DEHP and DiNP metabolites (ΣDEHPm and ΣDiNPm) were calculated by adding the molar concentration of metabolites and multiplying the sum by the molar weights of the respective diesters (Frederiksen et al. 2010). For DEHP and DiNP, the ratio between the molar concentration of the primary metabolite (MEHP and MiNP, respectively) and the total molar concentration of all four metabolites was calculated as %MEHP and %MiNP, as a measure of an individual's pattern of metabolism of DEHP and DiNP (Joensen et al. 2012). In

this study we distinguish between low and high molecular weight phthalate diesters and their metabolites, where DEP, DiBP, DnBP and BBzP are low molecular weight (LMW) phthalates and DEHP, DOP and DiNP are high molecular weight (HMW) phthalates.

Reproductive hormone analyses

Serum was stored at –20°C until analysis, and subsequently analyzed in batches. Concentrations of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and sex hormone-binding globulin (SHBG) were measured by time-resolved fluoroimmunoassay (Delfia, Wallac, Turku, Finland). Total testosterone and estradiol were determined by radioimmunoassay (Coat-a-Count, DPC, Los Angeles, USA and Pantex, Santa Monica, USA). Inhibin-B was determined by a double antibody enzyme-immunometric assay (Oxford Bioinnovation or DSL Beckman, USA). Between 2008 and 2010, slight adjustments were made to the inhibin-B analysis, and LOD was lowered from 20 to 7 pg/mL. The intra- and inter-assay coefficients of variation (CV) for measurement of FSH, LH, and SHBG were < 6%, and CVs for total testosterone were < 10%. Intra-and inter-assay CVs for estradiol were 8 and 13%, and for inhibin-B the CVs were 15 and 18%. We calculated free testosterone (cFT) from total testosterone and SHBG assuming a fixed albumin level of 43.8 g/L as described by Vermeulen (Vermeulen et al. 1999). Hormone ratios were calculated by simple division.

Semen analysis

Semen volume was assessed by weight, and sperm concentration was determined using a Bürker-Türk haemocytometer. Total sperm count [semen volume x sperm concentration] and percentage of progressively motile spermatozoa [WHO class A + B] were calculated (World Health Organization 2010). Morphology slides were fixed, Papanicolaou-stained, and assessed

according to strict criteria (Menkveld et al. 1990). Semen analysis was performed in accordance with WHO guidelines and has been described in detail previously (Jørgensen et al. 2002).

Statistics

Crude differences between the outcomes (phthalate metabolites, hormones, and semen quality variables) for *FLG* null and wild-type carriers were first analyzed using the Mann-Whitney test for non-normally distributed continuous variables. Associations between the *FLG* genotype and outcomes were then estimated with multivariate linear regression models. Dependent variables were natural logarithmic (ln) transformed (all phthalate metabolites, hormones, hormone ratios, and semen volume), cubic root transformed (sperm concentration, total sperm count, total normal spermatozoa), squared (progressively motile spermatozoa), or square root transformed (morphologically normal spermatozoa) to achieve normality of distribution of the residuals. Adjusted means and 95% CI were calculated by back-transformation of the estimates from the linear regression analyses.

Potential confounders were identified and included as model covariates if they were significant predictors of the outcome ($p < 0.05$) in a bivariate linear regression model. For ease of interpretation, if a covariate was a significant predictor of outcome in several models with hormone levels as outcome, then that predictor was included for all models with hormones as an outcome. Likewise, a significant predictor of concentration of several phthalate metabolites would be included in all models with phthalate metabolite levels as an outcome. In models of associations between hormone levels and the *FLG* genotype, the following confounders were included as continuous variables: body mass index (BMI), smoking (cigarettes per day), alcohol consumption (units consumed in the week prior to participation), time of blood sampling, and age. The type of assay was additionally included as a dichotomous variable in the inhibin-B

models. In models of associations between *FLG* genotype and semen variables, we adjusted for ejaculation abstinence time (for models of semen volume, sperm concentration, and total sperm count, which were positively associated with abstinence time) or time from ejaculation to semen analysis (for models of the percentage of progressively motile spermatozoa, which was negatively associated with time from ejaculation to analysis). Percentage of morphologically normal spermatozoa was left unadjusted, as none of the tested confounders were significant predictors of the outcome ($p > 0.05$). Models of phthalate metabolite levels and *FLG* genotype were adjusted for age, number of cigarettes per day, and year of participation. Participants evaluated in 2007 were more likely to carry null *FLG* alleles than participants tested in 2008 and 2009 (see Supplemental Material, Table S1), and mean urinary levels of some phthalate metabolites were also highest in 2007 (Supplemental Material, Figure S1). Year of participation was a significant predictor for several phthalate metabolites and was therefore included as a potential confounder in all models of associations between *FLG* genotype and urinary phthalate metabolites.

There were no missing data for the *FLG* genotype or the reproductive hormones. Missing data for other variables of interest are presented in Table 2, and cases with missing data were excluded on an analysis-by-analysis basis.

Other confounders were considered but not included in the final model because they were not significant predictors of outcomes in the final models with serum hormones or semen quality as outcomes: ethnicity, BMI squared, birth weight, *in utero* exposure to tobacco smoke, previous or current diseases (varicocele, cryptorchidism, or sexually transmitted diseases), recent fever, recent use of medication, and season. BMI, time of day of urine sample delivery and alcohol intake were considered but were not generally significant predictors in models with urinary

phthalate metabolites outcomes. A p-level below 0.05 was regarded as statistically significant in all statistical tests and models. Data analysis was performed using PASW Statistics v.18 (IBM, New York, USA).

Results

Stratification by FLG genotype

A total of 65 (7.5%) men were either heterozygous (n = 63) or homozygous/compound heterozygous (n = 2) for *FLG* loss-of-function variants (Table 3). These 65 men thus constituted the "filaggrin null carrier" genotype group. The observed genotype prevalence of the 2282del4 and 2447X polymorphisms did not deviate significantly from the expected prevalence under the Hardy-Weinberg equilibrium assumption (p = 0.55 and p = 0.86, respectively), whereas R501X differed significantly (p = 0.03) based on a single homozygous carrier (the calculated expected number was 0.13). The two men with homozygous null genotypes (one homozygous for R501X and one compound heterozygote for R501X and 2282del4) did not report atopic disease or use of medication that could be ascribed to atopic diseases, and their urinary phthalate concentrations, reproductive hormones, and semen quality parameters were unremarkable. There were no significant differences in reported previous urogenital diseases (cryptorchidism, hernia, sexually transmitted diseases, or varicocele) between the two genotype groups. The frequency of self-reported atopic diseases was largely comparable between the *FLG* genotype groups (wild-type vs. heterozygous or homozygous null genotype): any atopic disease (24% vs. 22%), allergy or hay fever (15% vs. 17%), asthma (12% vs. 11%), and eczema (1.4% vs. 1.5%). Moreover, the use of medication prior to participation did not differ between the genotype groups; however, only few young men reported medication use within the past three months (28 men used any antibiotics, 27 men used any asthma or allergy medication, 32 used any medication for skin

conditions - mostly acne medication, 28 used any analgesics, 16 used any other type of medication).

Age, alcohol use, and smoking (cigarettes per day) were not significantly associated with genotype group (Table 2). BMI was slightly lower in the *FLG* null group than in the wild-type group (median 21.9 versus 22.6 kg/m^2, p = 0.04).

FLG and reproductive hormones and semen quality

We found no significant association between the *FLG* genotype and the levels of reproductive hormones or semen quality.

Urinary phthalate metabolite concentrations and FLG genotype

All metabolites were detectable in urine in > 95% of participants, except MiNP and MOP (measurable in 49% and 34% of men, respectively). Urinary concentrations of MOP were very low. MCPP is a metabolite of di-n-octyl phthalate (DnOP), but also a minor metabolite of other phthalate diesters, making it difficult to relate this metabolite to exposure to any specific phthalate. Therefore, data on MOP and MCPP were not analyzed further. Phthalate metabolite concentrations and sums of diester phthalates by *FLG* genotype group are shown in Table 4. Urinary concentrations of phthalate metabolites for the whole group of men have previously been published (Joensen et al. 2012). All phthalate metabolites and sums of phthalate isomers and metabolites ($\Sigma DBP_{(i+n)}$, ΣDEHPm and ΣDiNPm) were correlated to each other (all p < 0.001).

FLG null allele carriers had higher excretion of all phthalate metabolites, both LMW and HMW phthalates, after adjusting only for urinary dilution by osmolality (Table 4). After adjusting for other relevant confounders (age, smoking and year of participation), this difference remained

significant for the LMW phthalates MiBP, MnBP, and MBzP (Table 4 and Figure 1). Differences between group means were substantial, e.g. MnBP was 33% higher (95% CI: 16, 51%) in *FLG* null allele carriers when compared to wild-type carriers. For the HMW phthalates, *FLG* null allele carriers had higher osmolality-adjusted levels for every measured metabolite and the sums of DEHP and DiNP metabolites, but none of the associations were statistically significant after adjustment for year of participation, age and cigarettes per day.

When the analyses were re-run including only men that were of ethnic European origin (participant and both of his parents born in a European country), estimates and p-values remained essentially unchanged (data not shown). Neither %MEHP nor %MiNP, which reflect phthalate metabolism, was associated with *FLG* genotype (data not shown).

Discussion

Young healthy men with common *FLG* null variants had significantly higher internal exposure to phthalate metabolites than men with wild-type *FLG* genotypes, as assessed by urinary phthalate metabolite concentrations. The higher phthalate levels in *FLG* null carriers could be due to increased exposure to cosmetic products, or enhanced penetration of chemicals across the skin barrier, or both. Use of skin moisturizers to treat xerosis may indeed be higher in *FLG* null carriers (Sergeant et al. 2009). However, *FLG* null carriers had higher levels both of metabolites of LMW phthalates, commonly found in skin care products, and of HMW phthalates that are usually not present in personal care products. Urinary levels of LMW phthalates were higher than levels of HMW phthalates, which is in accordance with studies in other populations (reviewed in Wittassek et al. 2011). The median urine concentration of MEP, which is the primary metabolite of the LMW phthalate DEP and the most frequently detected phthalate in

personal care products, was considerably higher in the *FLG* null variant group than in other men. However, after adjustment for urinary dilution, year of participation, age and cigarette smoking, the association was not significant, in contrast with MnBP and MiBP which are metabolites of DBP isomers. A study on topical application of DEP and DBP showed that urinary concentrations of the metabolite MEP were much higher after dermal application than MnBP concentrations, that MEP was cleared more quickly than MnBP, and that compared with DBP more of the applied amount of DEP was recovered in the urine (as MEP) (Janjua et al. 2008). This supports the notion that enhanced permeability to DBP, and not only the increased exposure, may account for the difference in metabolite levels between the *FLG* genotype groups. Although we adjusted for confounding by several factors, the possibility of chance associations due to multiple testing or bias due to uncontrolled confounding cannot be ruled out. In addition, as we are not aware of similar studies, we cannot determine whether our findings would be consistent with other populations.

No significant differences in reproductive hormones or semen quality were found between *FLG* null and wild-type carriers. We cannot exclude, however, that *FLG* null carriers may also have a higher internal exposure to other factors, such as vitamin D, that may actually improve semen quality (Blomberg et al. 2011; Thyssen et al. 2012a).

We recently published associations between reproductive hormones and the proportions of DEHP and DiNP excreted as their respective primary metabolites (%MEHP and %MiNP, respectively) in this population. Several reproductive hormones were significantly associated with the proportions of %MEHP and %MiNP in that study, but there was little evidence of significant associations of urinary phthalate metabolites or sums of phthalates with reproductive hormones or semen quality (Joensen et al. 2012). In the present study, neither %MEHP nor

%MiNP was associated with *FLG* null genotype, suggesting that an individual's pattern of metabolism of DEHP and DiNP was unrelated to *FLG* null genotype.

Since phthalate diesters are rapidly metabolized and the metabolites are excreted mainly in the urine with elimination half-lives of usually less than 24 hours (Koch et al. 2005), the measured phthalate levels are reflective of very recent exposure to the parent compounds (phthalate diesters). Measurement of metabolites in urine samples with the methods we used is accepted as the state-of-the-art method for biomonitoring these substances. Despite the considerable day-to-day variation in excretion of phthalates, a single urine sample is reasonably predictive of exposure during a 3-month period (Hauser et al. 2004), which constitutes a reasonable exposure time to investigate effects on spermatogenesis and reproductive hormones. The *FLG* null carrier frequency of 7.5% was consistent with previous Danish population-based studies (Thyssen et al. 2010). The questionnaires focused on reproductive disorders and not skin or atopic disorders; hence we did not ask specifically for information about eczema, or use of medication for skin conditions that could be related to filaggrin deficiency. This may explain why the two homozygous individuals did not report such disorders, as well as the very low prevalence of self-reported atopic dermatitis of 1.4% in the whole group. Although we did not find any significant associations between the *FLG* genotype and reproductive hormone levels or semen quality parameters, the number of *FLG* null carriers in our population may have been too small to provide sufficient statistical power to detect small differences in these outcomes.

Our findings suggest that men with *FLG* loss-of-function alleles may be at risk of excessive chemical exposure due to differences in skin barrier function resulting from reduced *FLG* expression. The use of the phthalates DEHP, BBzP and DnBP in the European Union is currently restricted but not banned. Although these phthalates are classified as reproductive toxicants, the

use of DiNP is only restricted in certain toys and childcare articles for children under 3 years of age (European Chemical Agency 2010). Immunotoxicological effects have been proposed for some phthalates, but it is currently unknown whether phthalate exposure may increase the risk of allergic disorders (Kimber and Dearman 2010). While a recent cross-sectional study from Denmark showed that the prevalence of *FLG* null variants increased from 12.9% in individuals with atopic dermatitis born in 1936-1949 to 19% among individuals with atopic dermatitis born in 1976-1988 (Thyssen et al. 2012b), one may speculate whether the increased phthalate exposure from skin care products over the second half of the 20th century may have played a role in the epidemic of atopic dermatitis. In fact, a British group has proposed the "hapten-atopy" hypothesis which suggests that an increased exposure to chemicals, particularly during pregnancy and the first year of life, may predispose to subsequent development of atopic disease (McFadden et al. 2009). At present, clinical trials are being conducted to test the possibility of primary prevention of atopic disorders by using topical therapy with moisturizers (Irvine et al. 2011), but many such skin care products are not globally regulated by legislation and may contain phthalates (European Chemical Agency 2010). *FLG* null carriers may constitute a susceptible group in which special attention to phthalates and other dermally applied chemicals in skin care products is warranted.

Conclusions

Young healthy men with *FLG* null variants had significantly higher internal exposure to phthalate metabolites than wild-type carriers, and the results from this study raise important questions. Our findings must be replicated in other study populations, and additional work also would be necessary to determine whether higher levels of urinary phthalates reflect greater external exposure (due to increased use of skin care products), greater internal exposure (due to

increased absorption through the skin), or both. Moreover, it is unknown whether the possible increased skin permeability of *FLG* null carriers applies to classes of chemicals other than the phthalates. The question of a possible disruption of testicular function in *FLG* null carriers cannot with certainty be answered by this study. Also, the possibility of an increased uptake of dermally applied pharmaceuticals has never been investigated. This study underlines the importance of ensuring that excess exposure to potentially endocrine disrupting or otherwise harmful chemicals is not overlooked as a side-effect of the treatment aiming to restore a defective skin barrier.

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Table 1. Overview of phthalate abbreviations."Parent" phthalate diesters to which humans are exposed (left column), and their metabolites measurable in urine (right column).

Phthalate group	Phthalate diesters		Measured urinary metabolites		LOD (ng/mL)
LMW	DEP	Di-ethyl phthalate	MEP	Mono-ethyl phthalate	0.46
	DnBP	Di-n-butyl phthalate	MnBP	Mono-n-butyl phthalate	0.57
	DiBP	Di-iso-butyl phthalate	MiBP	Mono-iso-butyl phthalate	0.55
	BBzP	Butylbenzyl phthalate	MBzP	Monobenzyl phthalate	0.25
HMW	DEHP	Di(2-ethylhexyl) phthalate	MEHP	Mono-(2-ethyl-hexyl) phthalate	0.63
			MEHHP	Mono(2-ethyl-5-hydroxyhexyl) phthalate	0.30
			MEOHP	Mono(2-ethyl-5-oxohexyl) phthalate	0.30
			MECPP	Mono(2-ethyl-5-carboxypentyl) phthalate	0.17
	DOP	Di-octyl phthalate	MOP	Mono-octyl phthalate	0.15
			MCPP[a]	Mono-3-carboxypropyl phthalate	0.14
	DiNP	Di-iso-nonyl phthalate	MiNP	Mono-iso-nonyl phthalate	0.15
			MHiNP	Mono-hydroxy-iso-nonyl phthalate	0.12
			MOiNP	Mono-oxo-iso-nonyl phthalate	0.11
			MCiOP	Mono-carboxy-iso-nonyl phthalate	0.05

LMW: low molecular weight phthalates often found in personal care products.

HMW: high molecular weight phthalates used as plasticizers, infrequently found in personal care products.

[a]MCPP is a metabolite of DOP, but also a minor metabolite of DiOP, DiDP, DiNP, DEHP, BBzP and DBP.

Table 2. Basic characteristics, unadjusted serum concentrations of reproductive hormones, and semen quality parameters by *FLG* genotype in 861 men. Unadjusted median (5, 95th percentiles), regression coefficients from final multivariate models, and corresponding p-values from these models.

Variable	Missing (N)	*FLG* null allele carriers	*FLG* wild-type	Regression coefficients (95% CI)	p[a]
Age (years)	4	19 (19, 21)	19 (18, 22)		0.1[f]
BMI (kg/m^2)	7	21.9 (19, 28)	22.6 (19, 29)		0.04[f]
Alcohol use (units last week)	34	14 (0, 49)	12 (0, 42)		0.9[f]
Smoking (cigarettes/day)	13	0 (0, 15)	0 (0, 20)		0.5[f]
Urinary osmolality (Osm/kg)	0	0.9 (0.4, 1.1)	0.9 (0.3, 1.1)		0.07[f]
Testosterone (nmol/L)	0	19 (12, 31)	19 (11, 30)	-0.01 (-0.08, 0.07)[b]	0.9[g]
Estradiol (nmol/L)	0	75 (48, 127)	77 (45, 126)	0.01 (-0.07, 0.09)[b]	0.7[g]
SHBG (nmol/L)	0	29 (18, 50)	27 (12, 51)	-0.09 (-0.19, 0.01)[b]	0.07[g]
LH (IU/L)	0	3.1 (1.4, 6.4)	3.0 (1.4, 6.2)	-0.07 (-0.18, 0.04)[b]	0.2[g]
Inhibin-B (pg/mL)	0	188 (81, 298)	175 (98, 304)	-0.03 (-0.13, 0.07)[b]	0.6[g]
FSH (IU/L)	0	2.7 (1.2, 5.8)	2.3 (0.9, 5.6)	-0.14 (-0.28, 0.01)[b]	0.07[g]
cFT (ng/dL)	0	12 (7.9, 20)	12 (7.6, 21)	0.02 (-0.07, 0.10)[b]	0.7[g]
cFT/LH ratio	0	3.7 (1.9, 7.8)	4.1 (2.0, 8.6)	0.08 (-0.03, 0.20)[b]	0.2[g]
Testosterone/estradiol ratio	0	262 (150, 389)	242 (149, 382)	-0.02 (-0.09, 0.05)[b]	0.6[g]
Testosterone/LH ratio	0	5.5 (2.9, 12)	6.2 (3.0, 12)	0.06 (-0.04, 0.17)[b]	0.2[g]
Inhibin-B/FSH ratio	0	70 (12, 197)	79 (19, 303)	0.11 (-0.11, 0.33)[b]	0.3[g]
Semen volume (mL)	6	3.1 (1.3, 5.8)	3.2 (1.3, 6.3)	1.29 (1.16, 1.42)[b]	0.8[h]
Sperm concentration (10^6/mL)	3	48 (1.9, 172)	48 (4.4, 167)	3.95 (3.65, 4.26)[c]	0.8[h]
Total sperm count (10^6)	7	164 (5.6, 447)	142 (13, 537)	6.11 (5.66, 6.55)[c]	0.6[h]
Total normal spermatozoa (10^6)	19	11 (0.5, 58)	10 (0.1, 55)	2.6 (2.33, 2.87)[c]	0.4[h]
Morphologically normal (%)	19	7.0 (1.0, 17.0)	6.8 (0.5, 17.0)	2.64 (2.39, 2.89)[d]	0.5[i]
Progressively motile (%)	10	64 (28, 81)	61 (28, 80)	3942 (3524, 4361)[e]	0.4[j]

[a]p-values are for difference between genotype groups tested by multivariate linear regression. [b]Dependent variables were ln transformed. [c] Dependent variables were cubic root transformed. [d]Dependent variable was square root transformed. [e]Dependent variable was squared. [f]Mann-Whitney test. [g]Adjusted for body mass index (BMI), smoking (cigarettes per day), alcohol consumption (units consumed in the week prior to participation), time of blood sampling, and age. Type of assay was additionally adjusted for in the inhibin-B model. [h]Adjusted for ejaculation abstinence time. [i]Unadjusted. [j]Adjusted for time from ejaculation to semen analysis.

Table 3. Genotype distribution in 861 men from the general population. N (%).

Genotype	Homozygous	Heterozygous	Wild-type
R501X	1 (0.1)	21 (2.4)	839 (97.4)
2282del4	0	34 (3.9)	827 (96.1)
R2447X	0	10 (1.2)	851 (98.8)
FLG null genotype	2 (0.2)[a]	63 (7.3)	796 (92.5)

[a]One person was compound heterozygote for R501X and 2282del4.

Table 4. Osmolality adjusted urinary concentrations of phthalate metabolites by *FLG* genotype (ng/mL), and estimated percentage difference between genotype groups with corresponding p-values, from final multivariate regression models.

Phthalate group	Phthalate metabolite	Osmolality adjusted median (5, 95th percentiles)		Estimated percent difference[a] (95% CI)	p[a]
		FLG null carrier	*FLG* wild-type		
LMW	MEP	116 (18, 751)	83 (17, 2202)	0.4 (-39, 39)	0.9
	MiBP	77 (31, 201)	61 (22, 171)	21 (4, 39)	0.02
	MnBP	42 (14, 118)	29 (9, 89)	33 (16, 51)	0.0002
	$\Sigma DBP_{(i+n)}$	156 (65, 383)	117 (42, 319)	26 (9, 42)	0.003
	MBzP	46 (14, 171)	37 (10, 157)	24 (0.2, 47)	0.05
HMW	MEHP	5 (1, 15)	4 (0.5, 17)	13 (-12, 37)	0.3
	MEHHP	29 (12, 79)	23 (8, 76)	16 (-5, 37)	0.1
	MEOHP	19 (7, 45)	15 (4, 55)	20 (-1, 41)	0.06
	MECPP	20 (7, 55)	16 (6, 53)	14 (-5, 33)	0.1
	MiNP	0.7 (0.1, 4)	0.6 (0.1, 5)	-3 (-36, 30)	0.8
	MHiNP	6 (1, 26)	4 (0.9, 22)	25 (-2, 52)	0.07
	MOiNP	3 (0.7, 13)	2 (0.4, 11)	26 (-4, 56)	0.09
	MCiOP	8 (3, 55)	8 (3, 38)	14 (-7, 35)	0.2
	ΣDEHPm	96 (37, 231)	80 (29, 271)	16 (-3, 35)	0.1
	ΣDiNPm	24 (7, 154)	21 (6, 95)	16 (-6, 38)	0.2

LMW: low molecular weight phthalates often found in personal care products.

HMW: high molecular weight phthalates used as plasticizers, infrequently found in personal care products.

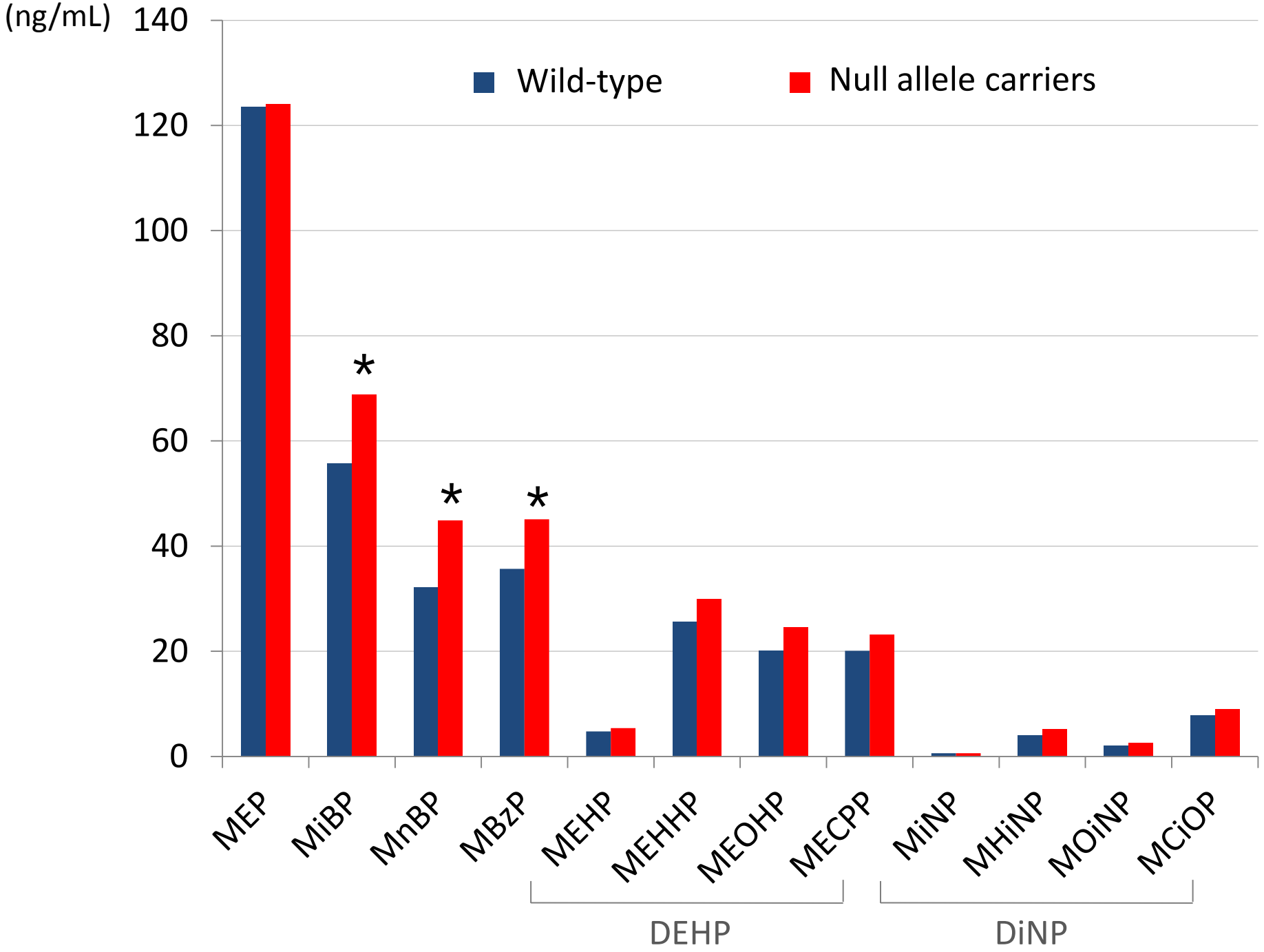
All shown metabolites were detectable in urine in > 95% of participants, except MiNP (measurable in 49% of men).

[a] Estimated percentage difference (95% CIs) and p-values are from multivariate linear regression models using ln transformed phthalate metabolite concentrations and adjusting for age, smoking and year of participation (adjusted means are depicted in Figure 2).

Figure Legend

Figure 1. Adjusted means of phthalate metabolites by *FLG* genotype. *FLG* null carriers (N = 65) had higher urinary excretion of common phthalate metabolites, from both LMW and HMW phthalates. Bars are geometric means (predicted values for 19 years, non-smoker, and participating year 2007).

* $p < 0.05$ for difference between genotype groups.


(ng/mL)
140
120
100
80
60
40
20
0
Wild-type
Null allele carriers
*
*
*
MEP
MiBP
MnBP
MBzP
MEHP
MEHHP
MEOHP
MECPP
MiNP
MHiNP
MOiNP
MCiOP
DEHP
DiNP